

Recognizing Phosphatidylinositol 3-Phosphate

Minireview

Saurav Misra, Gregory J. Miller,
and James H. Hurley¹

Laboratory of Molecular Biology
National Institute of Diabetes and Digestive
and Kidney Diseases
National Institutes of Health
Bethesda, Maryland 20892

Phosphatidylinositol 3-phosphate directs the endosomal localization of regulatory proteins by binding to FYVE and PX domains. New structures of these domains complexed with the phosphoinositide headgroup show how interactions with phosphate and hydroxyl groups differentiate this lipid from all others.

Lipids do things in signal transduction that other molecules can't. Lipid second messengers owe their special properties in cell regulation to their inability to diffuse unaided through the aqueous compartments of cells. Once synthesized in a particular cell membrane, a lipid messenger remains there as a marker for that membrane until the lipid is enzymatically converted or the membrane is budded off or fused with another. Stripped to its minimal components, a lipid messenger system contains an enzyme to synthesize the lipid messenger, one to degrade it, and at least one target to receive the signal. The simplicity and the membrane confinement of lipid messengers make them ideal regulators of subcellular localization.

There is an ever-growing class of proteins that are localized to specific cellular compartments by means of conserved domains that specifically recognize particular membrane-bound lipids (Hurley and Misra, 2000). Like the lipid messengers themselves, the protein domains that bind them are special. The crucial difference is that these protein domains recognize lipids embedded in phospholipid bilayers. Bilayers are a complex and dynamic sea. The interface region at the top of the bilayer contains headgroups, water molecules, ions, and polar backbone moieties. Deeper in the bilayer, the interface gives way to the hydrophobic core consisting of hydrocarbon tails. The boundaries between the aqueous phase, interface, and core are drawn with sharp lines in our cartoon (Figure 1), but in reality the boundaries are fuzzy. Membrane lipid binding domains recognize not only their specific partners, but they also interact with surrounding lipid molecules through much less specific electrostatic and hydrophobic interactions.

Of all lipid messengers, the phosphoinositides are the most versatile (Fruman et al., 1999; Czech, 2000; Simonsen et al., 2001). Phosphoinositides that are phosphorylated singly and in all possible combinations of the 3, 4, and 5 positions play roles in signaling and trafficking. For most of the 1990s, attention focused on PI(3,4,5)P₃, which is synthesized by the class I PI 3-kinases in response to receptor stimulation. In the last

few years, other phosphoinositides have begun to bask in the glow of attention. Many of these lipids are less acutely regulated and seem to be more important as determinants for spatial rather than temporal regulation. PI(3)P is a prime example of this newer wave of interest. It has attracted attention because it is a prominent and specific marker of endosomes. PI(3)P can be generated by two mechanisms: phosphorylation of PI by class III PI 3-kinase; and sequential hydrolysis of PI(3,4,5)P₃ by 4- and 5-phosphatases. Until recently, it was thought that PI(3)P was recognized by downstream targets through only one mechanism, by specific binding to FYVE domains within target proteins. We now know that PI(3)P action can proceed by binding to PX domains as well. Recognition of PI(3)P has been a challenge to explain in a structural sense because of the approximate 2-fold symmetry of the inositol ring. The arrangement of phosphate groups on PI(3)P is nearly equivalent to that of a PI(5)P molecule rotated by 180° about the axis between the 1- and 4- positions. The approximate symmetry is broken by differences in the stereochemistry of the free hydroxyls of the inositol ring, but until now structural studies of FYVE and PX domains have not explained why and how these differences mattered.

Two new papers in *Molecular Cell* provide the missing details and reveal how the special lipid of endosomes, PI(3)P, binds so specifically to the FYVE and PX domains. One structure shows how the PI(3)P headgroup, inositol (1,3)-bisphosphate (Ins(1,3)P₂), binds to the FYVE domain of the early endosome antigen-1 (EEA1; Dumas et al., 2001). The second shows how the PX domain of NADPH oxidase subunit p40^{phox} binds a soluble short-chain version of PI(3)P (Bravo et al., 2001).

How FYVE Domains Recognize PI 3-Phosphate

FYVE domains (found in Fab1p, YOTB, Vac1p, and EEA1) were the first cellular receptors for PI(3)P to be discovered (see references to the original reports by the Corvera, Emr, and Stenmark groups in Hurley and Misra, 2000; Gillooly et al., 2001). FYVE domains are double zinc fingers built of two small β sheets and a C-terminal α helix (Figure 1; Hurley and Misra, 2000). Their sequences contain a conserved (R/K)(R/K)HHCR motif that is crucial for PI(3)P binding. They are found in a variety of proteins involved in intracellular membrane transport processes, including endocytosis, early endosome fusion, and vacuolar transport (Gillooly et al., 2001). A second group of FYVE domain-containing proteins are found in signal transduction proteins, for example the SARA protein of the TGF- β receptor pathway (see references in Gillooly et al., 2001). This second group of FYVE proteins presumably directs signaling from endosomes. In contrast to PX domains, all FYVE domains that have been tested for phosphoinositide interaction exclusively bind PI3P and not to other phosphoinositides. FYVE domains are small (~80 residues) and their sequences are highly conserved. These factors sharply limit the range of potential lipid specificity of different FYVE domains (Hurley and Misra, 2000).

The FYVE domain story as it stood at the start of 2001 illustrated the remarkable inability of two high-resolution

¹Correspondence: jh8e@nih.gov

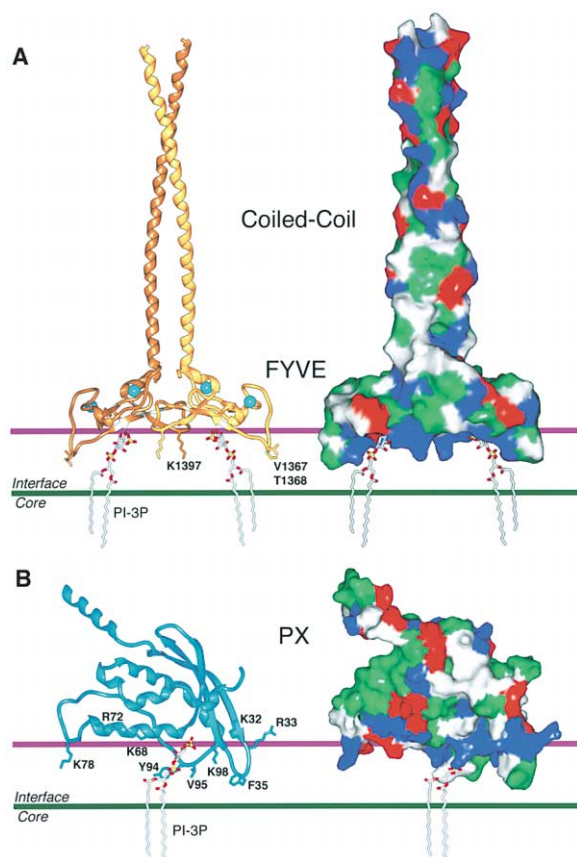


Figure 1. Schematic Drawing (left) and Molecular Surfaces (right) of EEA1-FYVE (A) and p40^{phox}-PX (B) Domains

The molecular surfaces are shown in the same orientation as the schematic drawings and are colored blue (basic), red (acidic), green (hydrophobic), and white (uncharged polar). The structures are docked to a membrane, with the polar interface and hydrophobic core regions of the membrane shown to scale. Residues predicted to be involved in nonspecific hydrophobic and electrostatic interactions with membrane lipids other than PI(3)P are shown. Myristoyl tails on PI(3)P have been modeled.

X-ray structure determinations to resolve the mechanism of action of a protein. The structures of the lipid-free FYVE domains from Vps27p and Hrs were determined by crystallography (Misra and Hurley, 1999; Mao et al., 2000). On the basis of the uncomplexed structure of the Vps27p FYVE domain, we suggested a model for PI(3)P binding (Misra and Hurley, 1999) in which the 3-phosphate interacted with the HHCR residues of the conserved motif as well as a conserved arginine on the fourth β strand. In this model, the 1-phosphate interacted with the (R/K)(R/K) at the start of the motif, all within with the same FYVE monomer. In this model, the hydrophobic tip of Vps27p-FYVE (corresponding to residues 1367-8 of EEA1 in Figure 1) of the FYVE domain inserted into the membrane. Hydrophobic insertion of part of the FYVE domain into the membrane is consistent with Lemmon's finding that FYVE domains strongly prefer to bind membrane-embedded PI(3)P, as opposed to the soluble headgroup (Sankaran et al., 2001). Quiocho and coworkers solved the structure of a FYVE domain dimer that was held together by citrate ions from the crystallization medium (Mao et

al., 2000). Making the reasonable guess that the polyanion citrate mimicked the binding of the PI(3)P headgroup, these authors proposed a radically different model. The PI(3)P binding site, although still formed by the conserved basic motif, is a shared property of the dimer, and therefore the monomer is incapable of binding PI(3)P in this model. The hydrophobic tip was proposed to form dimer contacts instead of penetrating the membrane. Membrane contacts were stabilized by a large, flat, basic face, instead of hydrophobic penetration. The extensive charged interactions predicted by this model would be consistent with the FYVE domain's strong preference for membrane-bound ligands. The differences in the models had implications for the cell biology of FYVE domains, since the first suggested that FYVE domains were not compulsory dimers, while the second suggested that they were.

Recent functional studies show that while FYVE domains often work as parts of dimeric proteins, they are not compulsory dimers. The isolated monomeric FYVE domain of EEA1 can bind PI(3)P vesicles in vitro with $\sim 2 \mu\text{M}$ affinity (Sankaran et al., 2001). The micromolar affinity of the monomer is insufficient for effective targeting to PI(3)P-containing membranes in cells (Gillooly et al., 2000). It seems likely that the concentration of PI(3)P in cells is too low to support monomer binding. A construct of two FYVE domains from Hrs in tandem, however, is targeted to PI(3)P-containing membranes (Gillooly et al., 2000). The explanation for the requirement for two FYVE domains lies in an avidity effect rather than in the direct dimerization of the FYVE domains. Some FYVE domain proteins may well work as monomers, with interactions other than FYVE-PI(3)P providing enough extra binding energy to drive translocation.

Recently, Overduin's group solved the NMR structure of the EEA1-FYVE monomer in the presence of PI(3)P micelles (Kutateladze and Overduin, 2001). Now, Lambright's lab (Dumas et al., 2001) has solved a high-resolution crystal structure of the EEA1 fragment consisting of part of the coiled-coil region and the FYVE domain, in complex with $\text{Ins}(1,3)\text{P}_2$. The EEA1 crystal structure shows that dimerization is mediated by extensive interactions between the coiled-coil domains (Figure 1A), consistent with previous functional work. The FYVE domains interact with each other in a much more limited manner. The EEA1 FYVE domains are rotated relative to each other by nearly 180° from their orientations in the Hrs FYVE dimer (Mao et al., 2000). Each FYVE monomer binds the $\text{Ins}(1,3)\text{P}_2$ in a manner similar to the model proposed for Vps27p and also observed in the NMR structure (Kutateladze and Overduin, 2001), effectively ruling out the dimeric recognition model (Mao et al., 2000). The $\text{Ins}(1,3)\text{P}_2$ binding mode and the coiled-coil dimerization of EEA1 restrict the orientation of the dimer relative to the membrane. The EEA1 FYVE domain orientation is similar, though more tilted relative to the membrane normal, to those proposed for the Vps27p FYVE domain or the NMR structure of the EEA1-FYVE monomer. There is likely to be some flexibility in the linkage between the FYVE domain and the rest of the protein, and it is not clear that there is a single "right" binding geometry for all FYVE domain/membrane interactions.

While the binding site and the general mode of PI(3)P headgroup binding in the new structure are similar to

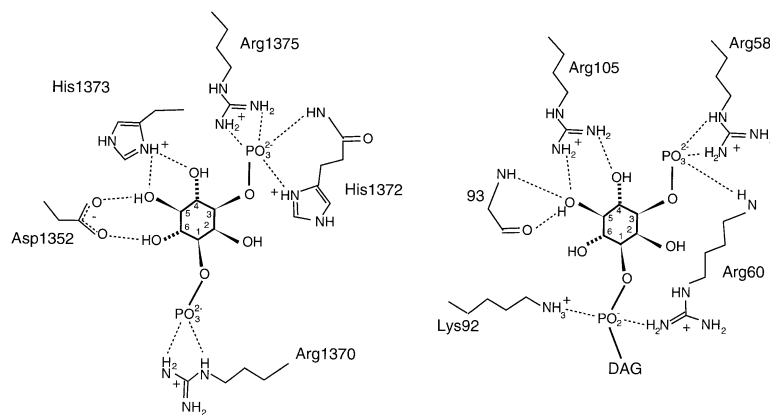


Figure 2. Schematic of PI(3)P Headgroup Recognition by FYVE (left) and PX (right)

Both domains rely on a combination of phosphate and hydroxyl moiety recognition to achieve stereospecificity.

some of the previous models, the new structure is the first definitive, high-resolution image of PI(3)P binding, and clarifies for the first time key aspects of stereospecific recognition. Hydroxyl interactions are the crux of discrimination between PI(3)P and PI(5)P, since the relative positioning of the phosphate groups is almost equivalent in these two phosphoinositides. A pair of hydrogen bonds is formed between the side chain of EEA1 Asp1352 and the 5- and 6- hydroxyls of PI(3)P (Figure 2). Mutation of the Asp only modestly decreases binding affinity, suggesting this residue is important mainly for selectivity against other phosphoinositides. The crucial role of this Asp was not predicted in the earlier models based on uncomplexed FYVE crystal structures, nor was this interaction observed in the NMR structure. The devil is in the details, and for details, nothing beats a high-resolution crystal structure of a relevant functional complex.

How PX Domains Recognize PI 3-Phosphate

PX domains were first identified in the N-terminal regions of p40^{phox} and p47^{phox}, two components of the phagocyte NADPH oxidase complex. The assembly of these proteins into an active complex on nascent phagosome membranes depends on both PI(3)P and PI(3,4)P₂. These compact ~130 amino acid domains have an $\alpha + \beta$ structure (Hiroaki et al., 2001) and occur in more than 150 eukaryotic proteins in addition to the phox proteins. Until this summer, nothing was known about the function of PX domains. We know now that many, and perhaps all, PX domains bind to PI(3)P, albeit with varying degrees of specificity. The surprise discovery of the PX domain as a second widespread PI(3)P binding module underscores that there is nothing in nature that requires a one-to-one correspondence between messenger molecules and their receiver modules.

The PX domains from the p47^{phox} (Kanai et al., 2001) and p40^{phox} (Ellson et al., 2001; Kanai et al., 2001) subunits of the NADPH oxidase complex, the sorting nexin SNX3 (Xu et al., 2001), the yeast t-SNARE Vam7p (Cheever et al., 2001), and cytokine-independent survival kinase (CISK; Virbasius et al. 2001) all bind PI(3)P. The PX domain of p47^{phox} preferentially binds PI(3,4)P₂ (Kanai et al., 2001), consistent with the cellular role of PI(3,4)P₂ in initiating recruitment of this protein to the nascent phagosome. Moreover, in the most comprehensive study so far, Lemmon and coworkers characterized the phosphoinositide binding specificities of all 15 of the

PX domains in the *Saccharomyces cerevisiae* genome and found that all bind PI(3)P (Yu and Lemmon, 2001). However, some of these yeast PX domains appear to bind other phosphoinositides as well. There is one report of a PX domain that does not bind PI(3)P, that of human C2-domain PI 3-kinase (CPK). The CPK-PX domain binds with low to moderate affinity to PI(4,5)P₂, although the biological relevance of possible CPK regulation by this phosphoinositide is unclear (Song et al., 2001).

The new structure determination of the p40^{phox}-PX domain bound to dibutanoyl PI(3)P (Bravo et al., 2001) beautifully reveals the determinants for stereospecific binding to this lipid. Following so closely on the heels of the discovery of the PI(3)P binding function of the PX domain, this is a very timely achievement. The 1- and 3-phosphate moieties are clamped by Arg side chains and main chain NH groups (Figure 2), much like what is seen in the phosphoinositide complexes with FYVE and other binding domains such as the pleckstrin homology (PH) domain (see references in Fruman et al., 1999; Czech, 2000; Hurley and Misra, 2000). The 4- and 5-hydroxyl groups are pinned down in an extensive hydrogen bond network with Arg-105 and with main chain residues in the polyproline region II (PPII)- α 3 loop. As also illustrated by the FYVE domain structure, most of the affinity is probably contributed by interactions with phosphates, but stereospecificity requires the involvement of the hydroxyl groups as well.

The structure contains two significant surprises. The p47^{phox} counterpart of Arg-57 is mutated in patients with chronic granulomatous disease, and it might have been thought that this Arg would therefore be involved in PI(3)P binding (see reference in Kanai et al., 2001). Indeed, Overduin and his coworkers found by NMR that PI(3)P binding induced a chemical shift change in this residue in Vam7p-PX (Arg-40 in Vam7p), and proposed a direct interaction on this basis (Cheever et al., 2001). The uncomplexed p47^{phox}-PX structure (Hiroaki et al., 2001) showed that this Arg has a structural role, but it seemed possible that the side chain could reposition in the complex. The p40^{phox}-PX crystal structure shows that even in the PI(3)P complex, the Arg-57 maintains its role in structural stabilization, but not in binding.

The second surprise is that the most conserved Arg in the binding site, Arg-105, is involved in hydroxyl interactions but not phosphate interactions. This observation creates a conundrum in terms of rationalizing the puta-

tive differences in phosphoinositide specificity between different PX domains. This Arg appears to be the major negative determinant that prevents 4- and 5-phosphorylated phosphoinositides from binding to p40^{phox}-PX. Phosphoryl groups attached to either the 4- or 5-hydroxyl would sterically collide with the Arg side chain, as well as disrupt the hydrogen bonds seen in the PI(3)P complex. Arg-105 is conserved in nearly all PX domains, including those that are reported to bind phosphoinositides other than PI(3)P. Some rationalizations for this apparent conflict are possible. As is customary when structural and functional data show apparent inconsistencies, putative conformational changes and different binding modes must be invoked. The region of the 4-hydroxyl is less confined than the region around the 5-hydroxyl. Modest shifts in the Arg side chain and the lipid might accommodate a 4-phosphoryl group. Accommodation of a 5-phosphoryl group in the same binding mode appears to be out of the question, so one might ask how PI(4,5)P₂ could possibly bind to a PX domain. Here, the PH domain field may offer a useful precedent (see references in Fruman et al., 1999; Hurley and Misra, 2000). PH domains come in a variety of flavors, including some such as phospholipase C- δ that bind specifically to PI(4,5)P₂, and others, such as BTK and Grp1 that bind with much higher affinity to PI(3,4,5)P₃. In the latter class of 3-phosphoinositide-specific PH domains, the 3-phosphoryl group occupies the same site that the 5-phosphoryl group of PI(4,5)P₂ occupies in the former class. Perhaps PI(4,5)P₂ can bind some PX domains by an analogous mechanism, in which the inositol ring is flipped with respect to the conformation seen in the p40^{phox}-PX complex.

It is not yet clear how strongly PX domains prefer membrane-bound versus soluble ligands. The substantial hydrophobic protrusions in the β 1- β 2 and PP_{II}- α 3 loops are consistent with significant membrane penetration on bilayer binding. The structure thus suggests that the PX domains are more like FYVE domains than PH domains in this respect. In recognition of the likely membrane association role of this region, the PP_{II}- α 3 loop has already been dubbed the "membrane attachment site" (Cheever et al., 2001). Less emphasis has been placed on possible membrane interactions involving the β 1- β 2 loop. This region is acidic in Vam7p-PX, but the p40^{phox}-PX structure shows that Phe-35 protrudes in a manner suggesting possible membrane penetration (Figure 1B). In most PX domains, the β 1- β 2 loop is predominantly hydrophobic and basic, so it may be premature to designate just one "membrane attachment site."

The specific binding and targeting of p40^{phox}-PX and related PX domains to PI(3)P-containing endosomes can safely be taken as fact. To the previous congruence of cellular and in vitro binding data, we can now add a high-quality structure that makes perfect sense in terms of all we know about phosphoinositide recognition and the specificity of this group of PX domains. The situation with PX domains that have been shown to bind other phosphoinositides in vitro is not as clear. Many of the studies have been done with filter binding assays, which are a very useful screening tool, but do not yield quantitative binding constants. With one or two important exceptions, the cellular rationales for the interactions with these other phosphoinositides are unclear. A number of

speculative assumptions are required to rationalize how they might bind to the site elucidated for p40^{phox}-PX. There is an urgent need to obtain higher quality in vitro binding data on this group as a class, and to determine the structures of PX domains bound to "alternative" phosphoinositides. Only after such studies are completed will we be able to say whether PX domains as a class are "FYVE-like" in recognizing primarily PI(3)P, as opposed to being more "PH-like" in recognizing a diverse array of phosphoinositides. Perhaps the PX domain will prove to be a unique entity not closely comparable to any other class. What is certain is that a significant new subfield of PX domain biology has just been launched.

Selected Reading

- Bravo, J., Karathanassis, D., Pacold, C.M., Pacold, M.E., Ellison, C.D., Anderson, K.E., Butler, P.J.G., Lavenir, I., Perisic, O., Hawkins, P.T., et al. (2001). *Mol. Cell* 8, 829–839.
- Cheever, M.L., Sato, T.K., de Beer, T., Kutateladze, T.G., Emr, S.D., and Overduin, M. (2001). *Nat. Cell Biol.* 3, 613–618.
- Czech, M.P. (2000). *Cell* 100, 603–606.
- Dumas, J.J., Merithew, E., Sudharshan, E., Rajamani, D., Hayes, S., Lawe, D., Corvera, S., and Lambright, D.G. (2001). *Mol. Cell* 8, 947–959.
- Ellison, C.D., Gobert-Gosse, S., Anderson, K.E., Davidson, K., Erdjument-Bromage, H., Tempst, P., Thuring, K.W., Cooper, M.A., Lim, Z.-Y., Holmes, A.B., et al. (2001). *Nat. Cell Biol.* 7, 679–682.
- Fruman, D.A., Rameh, L.E., and Cantley, L.C. (1999). *Cell* 97, 817–820.
- Gillooly, D.J., Morrow, I.C., Lindsay, M., Gould, R., Bryant, N.J., Gaullier, J.-M., Parton, R.G., and Stenmark, H. (2000). *EMBO J.* 19, 4577–4588.
- Gillooly, D.J., Simonsen, A., and Stenmark, H. (2001). *Biochem. J.* 355, 249–258.
- Hiroaki, H., Ago, T., Ito, T., Sumimoto, H., and Kohda, D. (2001). *Nat. Struct. Biol.* 8, 526–530.
- Hurley, J.H., and Misra, S. (2000). *Annu. Rev. Biophys. Biomol. Struct.* 29, 49–79.
- Kanai, F., Liu, H., Field, S.J., Akbary, H., Matsu, T., Brown, G.E., Cantley, L.C., and Yaffe, M.B. (2001). *Nat. Cell Biol.* 3, 675–678.
- Kutateladze, T., and Overduin, M. (2001). *Science* 291, 1793–1796.
- Mao, Y., Nickitenko, A., Duan, X., Lloyd, T.E., Wu, M.N., Bellen, H., and Quijcho, F.A. (2000). *Cell* 100, 447–456.
- Misra, S., and Hurley, J.H. (1999). *Cell* 97, 657–666.
- Sankaran, V.G., Klein, D.E., Sachdeva, M.M., and Lemmon, M.A. (2001). *Biochemistry* 40, 8581–8587.
- Simonsen, A., Wurmser, A.E., Emr, S.D., and Stenmark, H. (2001). *Curr. Opin. Cell Biol.* 13, 485–492.
- Song, X., Xu, W., Zhang, A., Huang, G., Liang, X., Virbasius, J.V., Czech, M.P., and Zhou, G.W. (2001). *Biochemistry* 40, 8940–8944.
- Virbasius, J.V., Song, X., Pomerleau, D.P., Zhan, Y., Zhou, G.W., and Czech, M.P. (2001). *Proc. Natl. Acad. Sci. USA* 98, 12908–12913.
- Xu, Y., Hortsman, H., Seet, L., Wong, S.H., and Hong, W. (2001). *Nat. Cell Biol.* 3, 658–666.
- Yu, J.W., and Lemmon, M.A. (2001). *J. Biol. Chem.*, in press.